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RESEARCH PAPER

An assessment of the *in vivo* efficacy of the glycogen phosphorylase inhibitor GPi688 in rat models of hyperglycaemia

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Background and purpose: Studies in cultured hepatocytes demonstrate glycogen synthase (GS) activation with glycogen phosphorylase (GP) inhibitors. The current study investigated whether these phenomena occurred *in vivo* using a novel GP inhibitor.
Experimental approach: An allosteric GP inhibitor, GPi688, was evaluated against both glucagon-mediated hyperglycaemia and oral glucose challenge-mediated hyperglycaemia to determine the relative effects against GP and GS *in vivo*.
Key results: In rat primary hepatocytes, GPi688 inhibited glucagons-mediated glucose output in a concentration dependent manner. Additionally GP activity was reduced and GS activity increased seven-fold. GPi688 inhibited glucagon-mediated hyperglycaemia in both Wistar (65%) & obese Zucker (100%) rats and demonstrated a long duration of action in the Zucker rat. The *in vivo* efficacy in the glucagon challenge model could be predicted by the equation; % glucagon inhibition $= 56.9 + 34.3$[log ([free plasma]/rat IC_{50})], $r = 0.921$). GPi688 also reduced the blood glucose of obese Zucker rats after a 7 h fast by 23%. In an oral glucose tolerance test in Zucker rats, however, GPi688 was less efficacious (7% reduction) than a glycogen synthase kinase-3 (GSK-3) inhibitor (22% reduction), despite also observing activation (by 45%) of GS *in vivo*.
Conclusions and implications: Although GP inhibition can inhibit hyperglycaemia mediated by increased glucose production, the degree of GS activation induced by allosteric GP inhibitors *in vivo*, although discernible, is insufficient to increase glucose disposal. The data suggests that GP inhibitors might be more effective clinically against fasting rather than prandial hyperglycaemic control.
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Abbreviations: AR-A025164, 3-amino-6-{3-fluoro-4-[(4-methylpiperazin-1-yl)sulphonyl]phenyl}-*N*-pyridin-3-ylpyrazine-2-carboxamide GP, glycogen phosphorylase; GPi688, 2-chloro-*N*-{1-[(2*R*)-2,3-dihydroxypropyl]-(3*R*/*S*)-2-oxo-1,2,3,4-tetrahydroquinolin-3-yl}-6H-thieno[2,3-b]pyrrole-5-carboxamide; GS, glycogen synthase; OGTT, oral glucose tolerance trial

Introduction

Type 2 diabetes is a severe and increasingly prevalent disease (World Health Organisation, 2006) characterized by altered glucose metabolism and insulin secretion. If hyperglycaemia is not well controlled, diabetes can result in increased cardiovascular complications (Keen *et al*., 1999). Current therapies do not achieve adequate glycaemic control, and so there is the need for more effective drugs (Tadayyon and Smith, 2003).

In severe diabetes, fasting hepatic glucose production is elevated compared to non-diabetic subjects (Reaven *et al*., 1988; Jeng *et al*., 1994; Hundal *et al*., 2000; Basu *et al*., 2004a). In patients with less severe hyperglycaemia, the situation is less clear. Compared to normal controls, moderate type 2 diabetic patients (fasting plasma glucose <8 mM) have no detectable increase in hepatic glucose production (Jeng *et al*., 1994; Staehr *et al*., 2002; Basu *et al*., 2004b). There is a derangement in both glucose-mediated suppression of prandial hepatic glucose production and hepatic glucose uptake in diabetic subjects (Mevorach *et al*., 1998). Thus,

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clinical data indicate that an improvement in hepatic glucose regulation is a viable target with which to treat both severe and less severe type 2 diabetes.

Glycogen phosphorylase (phosphorylase; EC 2.4.1.1; GP) is a key enzyme in the mobilization of glycogen. It catalyses the phosphorolysis of the glycosidic bond in glycogen to generate glucose-1-phosphate. Inhibition of GP has been proposed as a potential approach for the treatment of hyperglycaemia (Treadway *et al*., 2001). Indole-2-carboxamide and indole-2-glycine inhibitors of GP interact with the protein at a new allosteric binding site made up of the dimer interface (Rath *et al*., 2000; Wright *et al*., 2005). Compounds that bind at both this site and the AMP-binding site, not only result in allosteric inhibition of GP in a glucose-dependent manner (Hoover *et al*., 1998) but are also reported to increase the incorporation of gluconeogenic substrate as glycogen in hepatocytes (Shiota *et al*., 1997; Treadway *et al*., 2001). Additionally, an increase in glycogen synthesis and stimulation of hepatic glucose uptake in the dog, by fourfold, has been reported with the indole inhibitor CP316819 (Torres *et al*., 2003). Thus, there are potential additional actions of GP inhibitors, such as inhibition of the gluconeogenic-derived hepatic glucose output, and improvements of hepatic glucose uptake, which would enhance efficacy compared to a pure inhibitor of hepatic glucose output. In contrast, for those GP inhibitors that bind at the catalytic site of the enzyme, such as the imino sugar 1,4-dideoxy-1,4-imino-D-arabinitol, there is no evidence of either direct or indirect inhibition of gluconeogenesis in either hepatocytes, the perfused rat liver, in whole rats, or in conscious dogs following treatment (Fosgerau *et al*., 2001, 2002).

Compounds interacting at the indole site promote the tense- or T-state rather than the relaxed- or R-state of GP*a* (Oikonomakos *et al*., 1992). The phosphorylated enzyme, an endogenous inhibitor of glycogen synthase (GS) phosphatase, is a better substrate for the regulatory phosphorylase phosphatase PP1 when in the R-state (Bollen, 2001). Hence the phosphorylase inhibitor removes the inhibitory effect of GP*a* upon GS and so stimulates glycogen synthesis (Bollen *et al*., 1998). The indole inhibitor, CP91149 but not 1,4-dideoxy-1,4-imino-D-arabinitol, has been shown to inhibit GP, decrease the phosphorylation status of GP, increase GS activity and increase glycogen synthesis in rat primary hepatocyte (Latsis *et al*., 2002; Aiston *et al*., 2003b). CP91149 treatment increased translocation of the activated synthase to the particulate from the soluble cell fraction (Aiston *et al*., 2003a, b), effects mimicked by overexpression of the glycogen targeting subunit protein targeting to glycogen (Green *et al*., 2004).

There is limited information, however, for the presence of GS-induced activation following inhibition of GP *in vivo*. Increased GS flux but not GS activity in conscious, 13 weeks old, fasted zucker diabetic fatty (ZDF) rats was observed following GP inhibition with CP368296 (Shiota *et al*., 2005). Allosteric inhibition of GP, therefore, has the potential to inhibit hepatic glucose output through the direct inhibitory effect upon GP and may also have the potential to activate GS and glucose disposal as glycogen. This would have an effect upon disposal of glucose during a hyperglycaemic excursion and of storing of gluconeogenic-derived glucose-6-phosphate as hepatic glycogen. Thus, a phosphorylase inhibitor may, not only inhibit fasting-induced hyperglycaemia but also to improve the prandial hepatic glucose handling in type 2 diabetics.

With the exception of metformin, which is believed to inhibit hepatic gluconeogenesis, there is currently no agent available which has a direct action on the liver (Hundal *et al*., 2000). The current paper describes the *in vivo* properties of a novel GP inhibitor, GPi688 (2-chloro-*N*-{1-[(2*R*)-2,3-dihydroxypropyl]-(3*R*/*S*)-2-oxo-1,2,3,4-tetrahydroquinolin-3-yl}-6H-thieno[2,3-b]pyrrole-5-carboxamide), which binds at the indole site of the enzyme, and has been shown to be both selective for the liver vs muscle isoform of phosphorylase, and to be sensitive to the prevailing glucose and AMP concentrations (Freeman *et al*., 2006). We have used the compound to investigate whether GP inhibition can activate GS *in vivo* sufficient to enhance glucose disposal in an insulin-resistant Zucker rat. Our data suggest that the GS activation induced by allosteric GP inhibitors *in vivo* is insufficient to increase glucose disposal in the conscious Zucker rat.

Methods

All animal procedures were in strict accordance with the Animals (Scientific Procedures) Act of 1986 (UK).

In vitro *assays*

Cellular potency was measured in hepatocytes isolated by collagenase perfusion of liver from halothane-anaesthetized, male, Alderley Park Wistar rats (180–240 g body weight, AstraZeneca Biological Services, Alderley Park, Macclesfield, UK). The hepatocytes were cultured in monolayer overnight in the presence of dexamethasone, glucose and insulin. After replacement of the media with glucose-free Krebs–Henseleit solution, potency was assessed by inhibition of glucagon-mediated glucose output (Freeman *et al*., 2006). For determination of GP*a* and GS activities, in both hepatocytes and liver sample homogenates, incubations were terminated by snap freezing in liquid nitrogen. GP*a* activity was measured in the 13 000 *g* supernatant spectrophotometrically in the glycogenolytic direction (Aiston and Agius, 1999). GS activity was measured simultaneously in the rat hepatocytes by [1-^{3}H]-UDP-glucose incorporation into glycogen obtained from the cell lysate (Aiston *et al*., 2003a).

Protein binding was measured using the equilibrium dialysis technique based on the method described by Wan and Bergstroem (2007). GPi688 was added to plasma and dialysed with isotonic buffer for 18 h at 37 °C. The plasma and buffer solutions were analysed using generic liquid chromatography (LC)/ultraviolet (UV)/mass spectrometry (MS) and the percentage free determined in 100% plasma.

In vivo *assays*

Pharmacokinetics. GPi688 was dosed to Alderley Park Wistar rats (on a 12 h:12 h light–dark cycle and with free access to water and standard rat chow) either orally (20 μmol kg^{-1}, in

0.25% polyvinyl pyrrolidone (Kollidon 25, BASF, BTC Speciality Chemical Distribution, Cheadle Hulme, UK)/ 0.05% SDS (Sigma-Aldrich Chemicals, Poole, UK)) or intravenously (5 μmol kg^{-1}, in 25% hydroxypropyl β-cyclodextrin, Kleptose, HP, Roquette, Lestrem, France). Two animals were dosed per route and blood samples were obtained by tail-vein venepuncture for up to 24 h after dosing. Plasma compound concentration was measured by LC/MS. Plasma samples or calibration standards (100 μl) were vortex mixed with acetonitrile (200 μl) to precipitate the plasma proteins, the resulting mixture was centrifuged, and the supernatant decanted prior to injection (10 μl) onto the LC/MS system. Separation was achieved using a Prodigy 3 μm ODS(3), 100 mm × 4.6 mm, high-performance liquid chromatography column (Phenomenex, Macclesfield, UK), and a water/ acetonitrile/formic acid ratio of 40:60:0.2 mobile phase. Detection was by means of a Sciex API-365 detector. Calibration standards were prepared by adding methanolic solutions of known concentrations of GPi688 into plasma from undosed rats. The typical limit of quantification was 0.01 μM.

Pharmacodynamics. *In vivo* potency of GPi688 was measured in both Wistar and Zucker (*fa/fa*) male rats (AstraZeneca Biological Services) using a glucagon challenge to induce hyperglycaemia (Loxham *et al*., 2007). The efficacy of GPi688 was also assessed against an oral glucose tolerance test in obese Zucker rats.

Male Alderley Park Zucker rats (10–13 weeks old) or male Alderley Park Wistar rats (6–7 weeks old) both on a 08:00–20:00 h light cycle with *ad libitum* access to standard rat chow (RM1 for Wistar and RM3 for Zucker rats, Research Diets, New Brunswick, NJ, USA) were used to assess both *in vivo* potency and duration. GPi688 (up to 125 μmol kg^{-1}) or vehicle (0.25% polyvinyl pyrrolidone/0.05% SDS) was dosed in both strains of rat, a glucose reading was obtained with blood taken from the tail obtained by a pin prick (Roche Glucotrend hand-held monitor, Welwyn Garden City, UK) prior to glucagon challenge. Glucagon (200 μg kg^{-1}, s.c. (Peninsula Laboratories, Bachem, St Helens, UK) diluted in 0.85% physiological saline) was administered either at 90 min after compound administration for the dose–response studies; or at various times post-oral dose of GPi688 for determination of the duration of inhibition. Blood glucose readings were measured, by tail prick, at 45 min post-glucagon challenge and blood samples were taken, for pharmacokinetic (PK) analysis, via cardiac puncture, following death from CO_2, inhalation.

Oral glucose tolerance responses were measured in male obese Zucker rats following a 7 h fast. Either compound vehicle ($n = 12$), GPi688 (125 μmol kg^{-1}; $n = 8$) or the glycogen synthase kinase-3 inhibitor AR-A025164 (3-amino-6-{3-fluoro-4-[(4-methylpiperazin-1-yl)sulphonyl]phenyl}-*N*-pyridin-3-ylpyrazine-2-carboxamide) (10 mg kg^{-1}; $n = 8$) was administered orally 2 h before an oral glucose load (2 g kg^{-1}) was given. Blood glucose was measured before and up to 120 min after the glucose load. The liver was excised at the end of the protocol for the measurement of glycogen concentration. The left lateral lobe samples taken for analysis were snap frozen in liquid nitrogen and stored at −80 °C until analysed. Tissue glycogen concentration was analysed by KOH digestion of tissues at 70 °C followed by amyloglucosidase conversion of glycogen to glucose. The liberated glucose was measured by the addition of hexokinase and measurement of the absorbance of the NADPH generated from the subsequent reaction at 340 nm. Glycogen concentration in tissues was calculated as micromoles per gram tissue. In a separate group of animals, either vehicle or GPi688 was administered in the same way ($n = 5$ per group) and the animals killed 2 h later for measurement of GP and GS activities. In addition, another group of animals ($n = 5$ per group) were not only treated in the same way with either vehicle or GPi688 but also received glucose (2 g kg^{-1}, p.o.) 2 h later. These animals were killed after 20 min and their livers removed for GP and GS assays. Enzyme activity was measured using the same method as for the hepatocyte extracts.

Statistical analysis

Data are quoted as the mean ± s.e. of the mean, unless otherwise shown. Statistical analysis was undertaken by initial single factor analysis of variance followed by one-tailed *t*-test between treatment groups and the appropriate control group.

Materials

GPi688 was synthesized as described in patent WO 03/ 0745321A. AR-A025164 was synthesized as described in patent WO 03/004472.

Results

In vitro *potency and protein binding*

Hepatic GP activity was inhibited by approximately 45% following incubation with 1 μM of GPi688 (Figure 1a), a concentration that is close to the IC_{50} of the compound for inhibition of glucagon-induced glycogenolysis in the same hepatocyte preparation. At the same time, GS activity

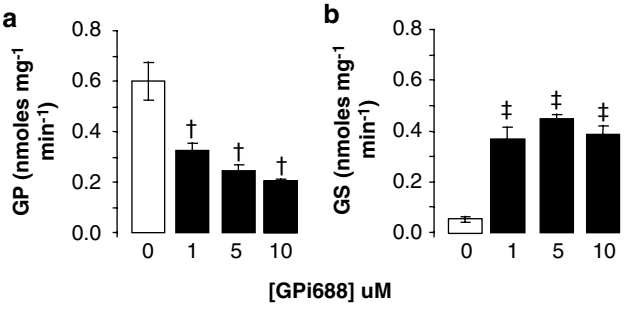

Figure 1 Effect of GPi688 upon glycogen phosphorylase (GP) and glycogen synthase (GS) enzyme activities in rat hepatocytes. GP (**a**) and GS (**b**) activities were measured in rat primary hepatocytes following incubation with GPi688 at varying concentrations for 60 min ($n = 3$). $^{\dagger}P < 0.0005$; $^{\ddagger}P < 0.0001$ vs control.

increased sevenfold (Figure 1b). Plasma protein binding (mean with 95% confidence limits) was higher for rat (0.35% free; 0.27–0.46%) than for human (1.22% free; 1.04–1.40%) serum.

In vivo *pharmacokinetic profile*

Oral dosing of GPi688 (20 μmol kg^{-1}) produced a good exposure profile in the Wistar rat (Figure 2). Peak concentration was seen at 4–6 h after dosing (14.5 and 19.4 μM) and declined to ~2.5 μM at 24 h. Following intravenous dosing of GPi688 (5 μmol kg^{-1}), peak plasma concentrations of 16.9 and 17.5 μM were observed at the first sample point (5 min). By the end of the time course, the compound concentration had fallen to around 0.4 μM in both rats. From these profiles, the bioavailability was assessed at 100%, a volume of distribution of 1.93 l kg^{-1}, plasma clearance of 2.4 ml min^{-1} kg^{-1} and a terminal half-life following intravenous dosing of 11.4 h.

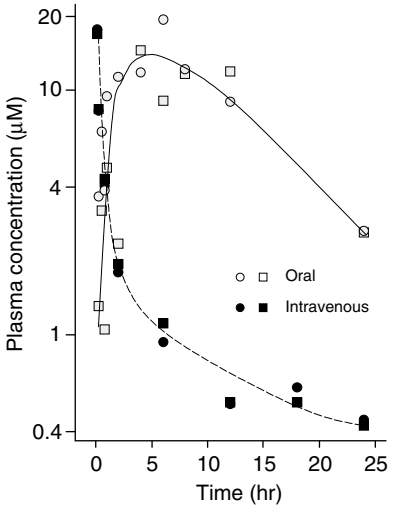

Figure 2 Pharmacokinetic profile of GPi688 in Wistar rats. GPi688 was formulated in either 0.25% polyvinyl pyrrolidone/0.05% SDS for oral dosing; or in 25% β-cyclodextrin for intravenous dosing. Two rats were dosed orally at 20 μmol kg^{-1} and two other rats were dosed intravenously at 5 μmol kg^{-1}. Blood samples were obtained by tail venepuncture following the animals being placed briefly in a hot box. Compound concentration was measured by liquid chromatography/mass spectrometry.

Efficacy and duration

Inhibition of glucagon-induced hyperglycaemia was used as a measure of GP activation *in vivo*. Subcutaneous injection of glucagon in the Wistar resulted in an increase in blood glucose (Figure 3a). GPi688 (12.5–125 μmol kg^{-1}) inhibited the glucagon-induced hyperglycaemia dose dependently. The two lower doses provide no significant inhibition but 125 μmol kg^{-1} resulted in 65.1% inhibition ($P<0.03$) compared to the vehicle-treated animals. In the Zucker rat, glucagon resulted in an increase in blood glucose in the vehicle-treated animals (Figure 3b). GPi688 (3.75–125 μmol kg^{-1}) also inhibited the glucagon response in the Zucker rat. The highest dose completely inhibited (103.7%; $P<0.0001$) the glucagon response. The lowest dose, 3.75 μmol kg^{-1}, resulted in a small (14.5%) but not significant reduction in response. The ED_{50} for GPi688 in the Zucker rat was calculated to be 19.5 μmol kg^{-1} (mean, with 95% confidence limits, 11.0–34.7).

The duration of action of the compound was measured in conscious Zucker rats given glucagon either before or after GPi688 at 19.5 or 125 μmol kg^{-1}. In the absence of compound, glucagon increased blood glucose to the same level in the high- and low-dose group, respectively. The lowest dose of GPi688 inhibited the hyperglycaemia response by 47.6% ($P<0.03$) at 4 h, but was not significant at either 12 h (21.9%) or 24 h (9.6%) post-dose. In contrast, 125 μmol kg^{-1} inhibited the response for the whole 24-h period (Figure 4).

Pharmacokinetic–pharmacodynamic relationship

Plasma samples were taken, for measurement of compound concentration, from each Zucker rat after the blood glucose response to glucagon had been measured. Using the plasma compound concentration, the plasma protein binding and the compound potency in rat GP, it was possible to determine the relationship between the free plasma compound concentration and efficacy (Figure 5). The data

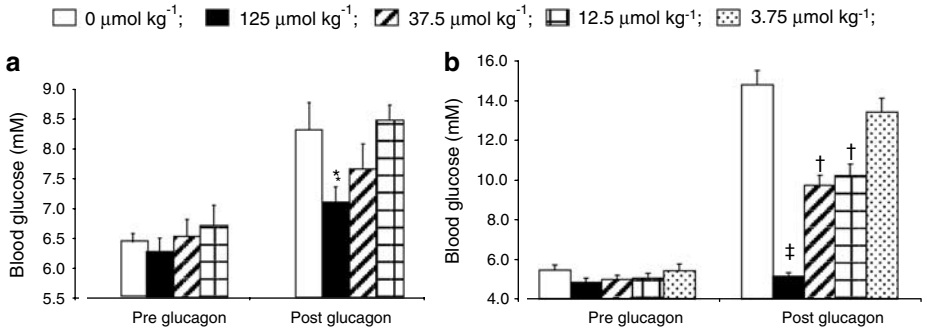

Figure 3 Acute *in vivo* efficacy of GPi688 in conscious rats. Male Wistar (**a**) or male Zucker (**b**) rats were injected with glucagon (200 μg/kg, s.c.) 45 min after dosing either vehicle (0.25% polyvinyl pyrrolidone/0.05% SDS), GPi688 at 3.75, 12.5, 37.5 or 125 μmol kg^{-1}, all p.o. Blood glucose was measured from a tail-prick sample using an Accuchek hand-held monitor. Each value is the mean ± s.e. of the mean, $n=4$–10, $^{*}P<0.03$; $^{\dagger}P<0.0001$, $^{\ddagger}P<0.00001$ vs control.

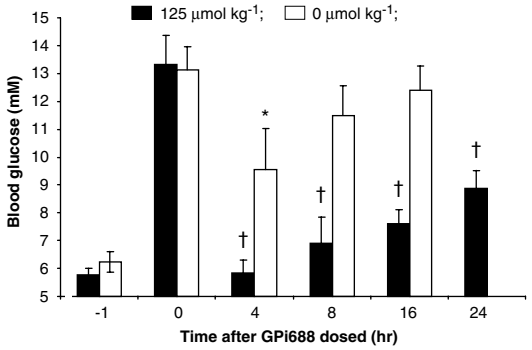

Figure 4 Duration and efficacy of GPi688 in the Zucker rat. Blood glucose was measured either before or 45 min after administration of glucagon (200 μg/kg, s.c.) in conscious, obese Zucker rats. The glucagon was given either after the administration of compound vehicle (time zero) or in GPi688-treated rats (125 or 19.5 μmol kg^{-1}, both p.o.) at either 4, 8, 12, 16 or 24 h after the compound. Each point is the mean ± s.e. of the mean, $n = 4$–6. $^{*}P < 0.03$; $^{\dagger}P < 0.001$ vs time zero values.

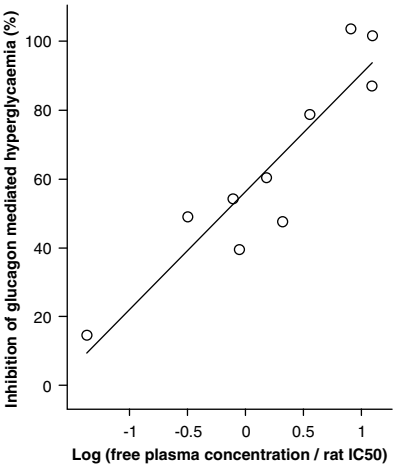

Figure 5 Pharmacokinetic–pharmacodynamic relationship of GPi688 in the Zucker rat. Blood samples were taken at the termination pharmacodynamic studies from both the acute efficacy (Figure 3b) and duration (Figure 4) paradigms. Plasma samples were analysed for concentration of GPi688, as described in the Methods. Using the values for protein binding in rat plasma (0.35%) and the IC_{50} value for rat liver enzyme (0.06 μM), it was possible to determine the free plasma concentration as a multiple of its potency against the rodent enzyme. When this parameter was expressed as the $\log arithm_{10}$, the relationship of the inhibition of the glucagons-mediated hyperglycaemia was best described by a straight line ($y = 56.9 + 34.3x$; $r = 0.921$). From this relationship, it was possible to determine that the free plasma drug concentration that was required to inhibit the glucagon response by 50% was 0.63 × rat enzyme IC_{50}, or 0.038 μM. Each point is the mean of four animals per time point and dose.

generated was best fitted with a straight line (percentage inhibition of glucagon = 56.9 + 34.3[log (free plasma concentration/rat IC_{50})], $r = 0.921$). The IC_{50} for GPi688 was determined as 0.038 μM, that is 0.63-fold the free plasma drug multiple (of 0.06 μM measured *in vitro*).

Oral glucose tolerance test

Two hours after oral administration, GPi688 (125 μmol kg^{-1}) increased hepatic GS by 54% compared to vehicle treatment (Figure 6a). In animals given the glucose load following GPi688, GS activity was not different from that in vehicle-treated animals receiving a glucose load. In rats where the blood glucose profile was measured, both the GPi688-treated and AR-A025164-treated animals had a lower fasting blood glucose concentration compared to that of vehicle-treated animals. The peak blood glucose response following glucose load was reduced only in the AR-A025164-treated rats (Figure 6c). Although the AUC response was 7.4% lower in the GPi688-treated animals (11.5 ± 0.3 mM min, $P < 0.04$) compared to vehicle (14.8 ± 0.5 mM min), AR-A025164-treated rats had a 24% reduction compared to vehicle (11.5 ± 0.3 mM min, $P < 0.0001$). Two hours following the glucose load, hepatic glycogen concentration was elevated only in the GPi688-treated animals (Figure 6b).

Discussion and conclusions

We have described the *in vivo* profile of GPi688, a novel GP inhibitor, which binds at the indole site of this enzyme (Whittamore *et al*., 2006). Oral bioavailability was 100% and the intravenous half-life was 11.4 h. This profile made the compound a good candidate for testing in a pharmacodynamic model. For this, we chose the glucagon-mediated hyperglycaemic response in the Wistar and obese Zucker rats. Glucagon induced a greater hyperglycaemia in the Zucker compared to the Wistar rat, which probably reflects the lack of a counter-regulatory effect of insulin in the insulin-resistant Zucker rat. GPi688 did not induce a hypoglycaemic response prior to administration of glucagon, consistent with the glucose-sensitivity effects (Martin *et al*., 1998), but induced a dose-dependent inhibition of the glucagon effect in both the rat strains. The oral potency of GPi688 was greater in the Zucker rat.

We observed that the efficacy of GPi688 in the glucagon challenge model could be described by a plot of the free plasma concentration as a multiple of the compound potency for the rodent enzyme. The free plasma concentration of GPi688 corresponding to 50% inhibition of the glucagon response was 38 nM, very close to the IC_{50} generated with the recombinant enzyme (61 nM; Freeman *et al*., 2006). All compounds assessed for *in vivo* activity in this way should have a position on the PK–PD plot dependent upon the relative partitioning of that compound between plasma and liver. Compounds with limited hepatic access will lie to the right, while those that partition preferentially into the liver will lie to the left. By measuring plasma compound concentration, knowing the plasma protein binding, the potency and assuming a specific position and slope of the PK–PD relationship for the particular class of compound, it is possible to determine the likelihood of *in vivo* activity.

Ercan-Fang *et al*. (2005) tried a similar approach when concluding that endogenous effectors modulated the *in vivo* efficacy of indole GP inhibitors. They found limited *in vivo* efficacy in relation to the measured potency in the presence of glucose alone, a 250- and 120-fold loss of potency in liver and mouse, respectively, was used as evidence for the presence of an, as yet unknown, endogenous modulator of

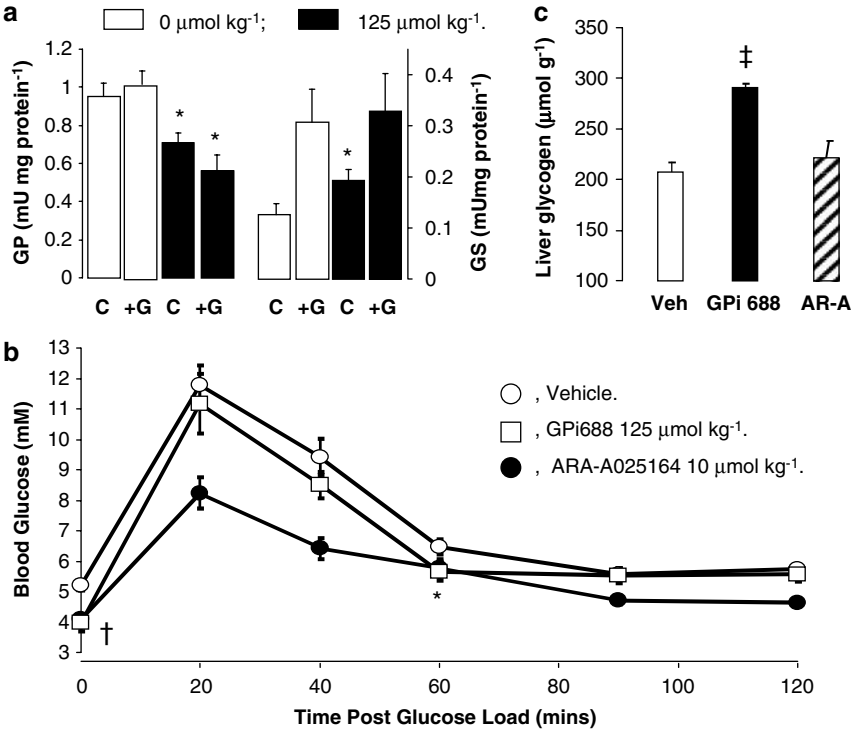

Figure 6 Effect of GPi688 on the oral glucose tolerance test (OGTT) in Zucker rats and upon enzyme activity in hepatocytes and liver. (**a**) Hepatic glycogen phosphorylase and synthase were measured *ex vivo* in obese Zucker rats 2 h following an OGTT. Rats were treated with either: vehicle followed by oral water (open bar, C; $n=5$); vehicle followed by oral glucose at 2 g kg^{-1} (open bar, +G; $n=5$); GPi688 125 μmol kg^{-1} followed by oral water (solid bar, C; $n=5$); or GPi688 125 μmol kg^{-1} followed by oral glucose at 2 g kg^{-1} (solid bar, +G; $n=5$). (**b**) OGTT was performed in obese Zucker rats following either vehicle (open circles; $n=12$), GPi688 (125 μmol kg^{-1}, open squares; $n=8$) or the GSK3 inhibitor AR-A025164 (10 mg kg^{-1}, black circles; $n=8$). (**c**) The liver glycogen concentration was measured *ex vivo* 2 h after the glucose challenge ($n=8$–10). Each point is the mean ± s.e. of the mean ($^{*}P<0.05$; $^{\ddagger}P<0.001$).

GP counteracting the compound. However, these authors failed to allow for the protein binding of CP316819. We have determined the plasma protein binding for CP316819 to be 98.2%. Taking into account the protein binding, the actual free compound concentration would be closer to 0.18 and 0.09 μM in the perfused liver and ob/ob mouse, respectively, which is much closer to the measured potency in the absence of modulators. In the current study, we measured GPi688 activity in the forward direction, using a glucose concentration producing approximately 30% inhibition of the liver GP*a* and ensuring adequate conversion to the T-state of the enzyme (Freeman *et al*., 2006). We believe that this is more representative of the physiological situation *in vivo*, and so, in conjunction with taking into account the free drug concentration, it gives a more accurate estimation for translating *in vitro* potency to *in vivo* efficacy.

Inhibition of GP, with indole site-binding compounds, can activate GS and glycogen synthesis in cultured rat primary hepatocytes (Aiston *et al*., 2003a, b; Latsis *et al*., 2002). We used GPi688 to explore the activation of GS with phosphorylase inhibitors in both rat primary hepatocytes that have been cultured overnight and *in vivo* using Zucker rats. In the rat primary hepatocyte, we observed activation of GS, measured 65 min after the start of incubation with GPi688, reflecting the inhibition of GP (Figure 1). Additionally, GS activity was increased, as measured 2 h after oral administration of GPi688, in the Zucker rat. Unlike in the hepatocyte where a fourfold increase was observed; however, the effect *in vivo* was a more modest 54% increase, while glucose alone produced a 2.5-fold increase over baseline. One possibility for the difference in GS response between the two paradigms was due to the time difference. It has been demonstrated, for instance, that the GS activity changes dynamically following glucose load, with an initial activation, followed by inhibition as the hepatic glycogen concentration changes (Niewoehner and Nuttall, 1995). It is possible therefore that there was in fact an initial, larger, activation of GS following exposure to GPi688. As the hepatic glycogen concentration increases, however, this results in inhibition of GS. This hypothesis is supported by the finding that hepatic glycogen concentration was increased by almost 50% compared to the vehicle-treated rats, when measured 4 h after treatment. In fasted ZDF rats, treatment with a GP inhibitor, although not resulting in an increase in GS at the time point evaluated, did increase GS flux and hepatic glycogen (Shiota *et al*., 2005). These last two parameters were taken as evidence of a functional benefit. Fasting plasma glucose in the Zucker rat was reduced in both GPi688- and AR-R025164-treated animals. The mechanism of action of GPi688 here could be either activation of glycogen synthesis or inhibition of hepatic glucose output (Figure 6c). The GS activation after the oral glucose tolerance trial (OGTT) was not enhanced by GPi688, which may account for the relative lack of efficacy in this model. In contrast, the glycogen synthase kinase-3

inhibitor, AR-A025164, was able to reduce plasma glucose both following fasting and the OGTT. Unfortunately, we do not have GS activity data in the AR-A025164-treated animals. Failure of GPi688 to activate GS was unlikely to be due to a failure to expose the animals to a maximally effective dose of the compound. The dose used was found to abolish completely the glucagon-mediated hyperglycaemia between 45 min and 4 h after dosing (Figures 3b and 4). We did not investigate the effects of GPi688 within 45 min of oral dosing. The T_{max} of AR-A025164 is 1.8 h (R. Bhat, personal communication, AstraZeneca Pharmaceuticals, Södertalje). We are confident, therefore, that each of the compounds used are either at the maximal effect (for GPi688) or at the maximal exposure (for AR-A025164) at the time when the glucose load was given.

It is intriguing that the hepatic glycogen concentration was not elevated following AR-R025164, in spite of AR-A025164 reducing the blood glucose profile by 22% with modest increase of hepatic glycogen. GPi688 reduced the glucose profile by only 7% despite a considerably larger effect upon hepatic glycogen concentration. It would appear that there might be different compartments of hepatic glycogen, GS and GP. The compartmentalization could be either intracellular (Fernandez-Novell *et al*., 1992; Green *et al*., 2004) or due to intra-hepatic distribution of the glycogen-metabolizing enzymes between periportal and perivenous hepatocytes (Jungermann and Kietzmann, 1996). The compartment in which glycogen is elevated by GP inhibitors may not be linked to plasma glucose elevated by an oral glucose tolerance test. Following a glucose load, the excess carbohydrate is taken up predominantly by the perivenous hepatocytes and stored as glycogen. Glycogen is then metabolized to lactate, which re-enters the circulation to the periportal cells and is converted to glycogen via gluconeogenesis. This hypothesis is consistent with data supporting an indirect route of hepatic glycogen synthesis, via lactate formation, found in studies using specifically labelled glucose in conscious rats (Newgard *et al*., 1983, 1984). It is known that indole inhibitors of GP can increase gluconeogenic substrate uptake and conversion to glycogen (Shiota *et al*., 1997; Treadway *et al*., 2001). It is possible that phosphorylase inhibitor treatment of animals in which an OGTT is performed enables increased hepatic glycogen synthesis from gluconeogenic substrate derived from the periportal glycogen, yet the rate of direct glucose incorporation into glycogen within the perivenous hepatocytes is unaltered. Hence, we observed a limited effect of GPi688 on blood glucose during the OGTT. The *ex vivo* measurement of GS activity that we employed was unable to differentiate between intra-hepatic and intracellular GS pools. A dissociation of hepatic glycogen and anti-hyperglycaemia actions has been described before (Yang and Newgard, 2003) in streptozotocin-treated rats that were treated with adenovirus to overexpress either the liver or a modified skeletal muscle glycogen targeting subunit. In this model, the expression of the liver isoform resulted in a much larger increase in hepatic glycogen concentration than seen with the modified muscle isoform, yet it was the latter that was more effective in reducing the hyperglycaemia.

We have reported previously that GPi688 is non-selective in *in vitro* assays when assessed at the same activity state of the phosphorylase α (Freeman *et al*., 2006). The *in vitro* potency of compounds, such as GPi688, is dependent upon the prevailing concentration of glucose or AMP in the liver or skeletal muscle isoform assay. Compound potency is increased with increasing glucose concentration and decreased by raising AMP concentration. Selectivity of GPi688 *in vivo* will be dependent upon the intracellular glucose and AMP concentrations, plus other as yet unknown regulators. We have not evaluated the *in vivo* selectivity of GPi688. There have been some studies investigating the acute effect of GP inhibition on the biochemical and functional responses during different muscle contraction paradigms in perfused rat hindquarters (Baker *et al*, 2005, 2006). These studies reported that prolonged aerobic contraction responses were suppressed while either acute or high-intensity responses were unaltered. It remains to be seen how responses are affected following chronic treatment in diabetic models.

The current study demonstrated a 50% increase in hepatic glycogen concentration within 4 h of dosing GPi688 (Figure 6). There are potential safety implications for the long-term inhibition of GP that need to be investigated further. If treatment with GP inhibitors produced a sustained elevation of hepatic glycogen, there may be the potential for adverse effects such as hepatomegaly. Subjects with Hers' disease, lacking an active liver isoform of GP, due to a mutation of the *PGLY* gene, display hepatomegaly, mild hypoglycaemia, hyperlipidaemia and ketosis (Burwinkel *et al*, 1998). Treadway *et al* (2001) have reported that the hepatic glycogen accumulation does not increase any further after the first 5 days of treatment with a GP inhibitor, but these authors did not present concentration levels of the GP inhibitor, measured over the dose interval. To our knowledge, no studies have been published on the toxicological implications of the long-term treatment with a GP inhibitor.

In summary, we have evaluated the *in vivo* profile of GPi688 against both pharmacokinetic parameters and pharmacodynamic efficacy in rodent models. Compound exposure following oral dosing was consistent with good oral bioavailability and when tested for inhibition of GP *in vivo* using the glucagon challenge, it was found to be potent and to give 24 h cover in the Zucker model following larger doses. By simultaneous measurement of plasma compound concentration from the different studies, and calculating the free concentration, we were able to demonstrate a positive relationship between free exposure and effect. Indeed, the free plasma exposure for a 50% effect *in vivo* was close to the IC_{50} measured *in vitro*. Although treatment with GPi688 was able to increase hepatic GS activity *in vivo*, as observed in isolated hepatocytes, the response was less than observed following the glucose load. As a result of the data obtained with GPi688, we do not believe that an allosteric inhibitor of GP is likely to increase prandial glucose disposal. GP inhibitors are more likely to be efficacious where the plasma glucose is derived through GP activation, such as in the fasted state. Clearly, there is still much that needs to be elucidated regarding the role of GP, and its interactions with other proteins, in the regulation of tissue glycogen and blood glucose.

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Conflict of interest

All authors are employees of AstraZeneca Pharmaceuticals.

References

Aiston S, Agius L (1999). Leptin enhances glycogen storage in hepatocytes by inhibition of phosphorylase and exerts an additive effect with insulin. *Diabetes* **48**: 15–20.

Aiston S, Andersen B, Agius L (2003a). Glucose 6-phosphate regulates hepatic glycogenolysis through inactivation of phosphorylase. *Diabetes* **52**: 1333–1339.

Aiston S, Coghlan MP, Agius L (2003b). Inactivation of phosphorylase is a major component of the mechanism by which insulin stimulates hepatic glycogen synthesis. *Eur J Biochem* **270**: 2773–2781.

Baker DJ, Greenhaff PL, MacInnes A, Timmons JA (2006). The experimental type 2 diabetes therapy glycogen phosphorylase inhibition can impair aerobic muscle function during prolonged contraction. *Diabetes* **55**: 1855–1861.

Baker DJ, Timmons JA, Greenhaff PL (2005). Glycogen phosphorylase inhibition in Type 2 diabetes therapy: a systematic evaluation of metabolic and functional effects in rat skeletal muscle. *Diabetes* **54**: 2453–2459.

Basu A, Shah P, Nielsen M, Basu R, Rizza RA (2004a). Effects of type 2 diabetes on the regulation of hepatic glucose metabolism. *J Invest Med* **52**: 366–374.

Basu R, Schwenk WF, Rizza RA (2004b). Both fasting glucose production and disappearance are abnormal in people with 'mild' and 'severe' type 2 diabetes. *Am J Physiol Endocrinol Metab* **287**: E55–E62.

Bollen M (2001). Combinatorial control of protein phosphatase-1 [review]. *Trends Biochem Sci* **26**: 426–431.

Bollen M, Keppens S, Stalmans W (1998). Specific features of glycogen metabolism in the liver [review]. *Biochem J* **336**: 19–31.

Burwinkel B, Bakker HD, Herschkovitz E, Moses SW, Shin YS, Kilimann MW *et al.* (1998). Mutations in the liver glycogen phosphorylase gene (Pygl) underlying glycogenosis type vi (Hers-disease). *Am J Hum Gen* **62**: 785–791.

Ercan-Fang N, Taylor MR, Treadway JL, Levy CB, Genereux PE, Gibbs EM *et al.* (2005). Endogenous effectors of human liver glycogen phosphorylase modulate effects of indole-site inhibitors. *Am J Physiol Endocrinol Metab* **289**: E366–E372.

Fernandez-Novell JM, Arino J, Vilaro S, Bellido D, Guinovart JJ (1992). Role of glucose 6-phosphate in the translocation of glycogen synthase in rat hepatocytes. *Biochem J* **288** (Pt 2): 497–501.

Fosgerau K, Breinholt J, McCormack JG, Westergaard N (2002). Evidence against glycogen cycling of gluconeogenic substrates in various liver preparations. *J Biol Chem* **277**: 28648–28655.

Fosgerau K, Mittelman SD, Sunehag A, Dea MK, Lundgren K, Bergman RN (2001). Lack of hepatic 'interregulation' during inhibition of glycogenolysis in a canine model. *Am J Physiol Endocrinol Metab* **281**: E375–E383.

Freeman S, Bartlett JB, Convey G, Hardern I, Teague JL, Loxham SJ *et al.* (2006). Sensitivity of glycogen phosphorylase isoforms to indole site inhibitors is markedly dependent on the activation state of the enzyme. *Br J Pharmacol* **149**: 775–785.

Green AR, Aiston S, Greenberg CC, Freeman S, Poucher SM, Brady MJ *et al.* (2004). The glycogenic action of protein targeting to glycogen in hepatocytes involves multiple mechanisms including phosphorylase inactivation and glycogen synthase translocation. *J Biol Chem* **279**: 46474–46482.

Hoover DJ, Lefkowitzsnow S, Burgesshenly JL, Martin WH, Armento SJ, Stock IA *et al.* (1998). Indole-2-carboxamide inhibitors of human liver glycogen phosphorylase. *J Med Chem* **41**: 2934–2938.

Hundal RS, Krssak M, Dufour S, Laurent D, LeBon V, Chandramouli V *et al.* (2000). Mechanism by which metformin reduces glucose production in type 2 diabetes. *Diabetes* **49**: 2063–2069.

Jeng CY, Sheu WH, Fuh MM, Chen YD, Reaven GM (1994). Relationship between hepatic glucose production and fasting plasma glucose concentration in patients with NIDDM. *Diabetes* **43**: 1440–1444.

Jungermann K, Kietzmann T (1996). Zonation of parenchymal and nonparenchymal metabolism in liver. *Ann Rev Nutr* **16**: 179–203.

Keen H, Clark C, Laakso M (1999). Reducing the burden of diabetes: managing cardiovascular disease [review] [27 refs]. *Diabetes Metab Res Rev* **15**: 186–196.

Latsis T, Andersen B, Agius L (2002). Diverse effects of two allosteric inhibitors on the phosphorylation state of glycogen phosphorylase in hepatocytes. *Biochem J* **368**: 309–316.

Loxham SJG, Teague J, Poucher SM, De Schoolmeester J, Turnbull AV, Carey F (2007). Glucagon challenge in the rat: a robust method for the *in vivo* assessment of glycogen phosphorylase inhibitor efficacy. *J Pharmacol Toxicol Met* **55**: 71–77.

Martin WH, Hoover DJ, Armento SJ, Stock IA, McPherson RK, Danley DE *et al.* (1998). Discovery of a human liver glycogen phosphorylase inhibitor that lowers blood glucose *in vivo*. *Proc Natl Acad Sci USA* **95**: 1776–1781.

Mevorach M, Giacca A, Aharon Y, Hawkins M, Shamoon H, Rossetti L (1998). Regulation of endogenous glucose production by glucose *per se* is impaired in type 2 diabetes mellitus. *J Clin Invest* **102**: 744–753.

Newgard CB, Hirsch LJ, Foster DW, McGarry JD (1983). Studies on the mechanism by which exogenous glucose is converted into liver glycogen in the rat. A direct or an indirect pathway? *J Biol Chem* **258**: 8046–8052.

Newgard CB, Moore SV, Foster DW, McGarry JD (1984). Efficient hepatic glycogen synthesis in refeeding rats requires continued carbon flow through the gluconeogenic pathway. *J Biol Chem* **259**: 6958–6963.

Niewoehner CB, Nuttall FQ (1995). Glycogen concentration and regulation of synthase activity in rat liver *in vivo*. *Arch Biochem Biophys* **318**: 271–278.

Oikonomakos NG, Acharya KR, Johnson LN. (1992). Rabbit muscle glycogen phosphorylase b. The structural basis of activation and catalysis. In: Crabbe MJC, Harding JJ (eds). CRC Press, Roca Baton. *Post-translational Modification of Proteins*, pp 81–151.

Rath VL, Ammirati M, Danley DE, Ekstrom JL, Gibbs EM, Hynes TR *et al.* (2000). Human liver glycogen phosphorylase inhibitors bind at a new allosteric site. *Chem Biol* **7**: 677–682.

Reaven GM, Hollenbeck C, Jeng CY, Wu MS, Chen YD (1988). Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with NIDDM. *Diabetes* **37**: 1020–1024.

Shiota M, Jackson PA, Bischoff H, Mccaleb M, Scott M, Monohan M *et al.* (1997). Inhibition of glycogenolysis enhances gluconeogenic precursor uptake by the liver of conscious dogs. *Am J Physiol Endocrinol Metab* **36**: E868–E879.

Shiota M, Torres TP, Fujimoto Y, Donahue EP, Howell JM (2005). Glycogen phosphorylase inhibitor (GPI) decreases net hepatic glucose production (NHGP) by increasing glycogen synthesis (HGS) in Zucker diabetic fatty rats (ZDF). *Diabetes* **54** (Suppl 1): A147.

Staehr P, HotherNielsen O, BeckNielsen H (2002). Hepatic glucose production: therapeutic target in type 2 diabetes? [review] [85 refs]. *Diabetes Obes Metab* **4**: 215–223.

Tadayyon M, Smith SA (2003). Insulin sensitisation in the treatment of type 2 diabetes [review] [113 refs]. *Expert Opin Investig Drugs* **12**: 307–324.

Torres TP, Sasaki N, Fujimoto Y, Lacy B, Cherrington AD, Treadway JL *et al.* (2003). Inhibition of glycogen phosphorylase suppresses basal and glucagon-induced glucose production and increases glucose uptake in the liver of conscious dogs. *Diabetes* **52** (Suppl 1): 1484-P.

Treadway JL, Mendys P, Hoover DJ (2001). Glycogen phosphorylase inhibitors for treatment of type 2 diabetes mellitus [review]. *Expert Opin Investig Drugs* **10**: 439–454.

Wan H, Bergstroem F (2007). High throughput screening of drug–protein binding in drug discovery. *J Liq Chromatogr Relat Technol* **30**: 681–700.

Whittamore PRO, Addie MS, Bennett SNL, Birch AM, Butters M, Godfrey L *et al*. (2006). Novel thienopyrrole glycogen phosphorylase inhibitors: synthesis, *in vitro* SAR and crystallographic studies. *Bioorg Med Chem Lett* **16**: 5567–5571.

World Health Organisation. Diabetes. Factsheet no 312. (2006).

Wright SW, Rath VL, Genereux PE, Hageman DL, Levy CB, McClure LD *et al*. (2005). 5-Chloroindoloyl glycine amide inhibitors of glycogen phosphorylase: synthesis, *in vitro*, *in vivo*, and X-ray crystallographic characterization. *Bioorg Med Chem Lett* **15**: 459–465.

Yang R, Newgard CB (2003). Hepatic expression of a targeting subunit of protein phosphatase-1 in streptozotocin-diabetic rats reverses hyperglycemia and hyperphagia despite depressed glucokinase expression. *J Biol Chem* **278**: 23418–23425.